

PHYSIOLOGICAL AND  
MORPHOLOGICAL IDENTIFICATION OF HORIZONTAL,  
BIPOLAR AND AMACRINE CELLS IN  
GOLDFISH RETINA

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SUMMARY

1. Intracellular recordings were made from various types of cells in the isolated goldfish retina, and Procion Yellow was injected from the recording pipette in order to identify histologically the structure recorded. The dye diffused and stained the cell body and processes down to the fine branches.

2. S-potentials were identified as coming from the external horizontal cells and from the internal horizontal cells. Both L- and C-type S-potentials were found in both regions, and no histological differences were seen in cells giving these two types of responses. S-potentials recorded from the external horizontal cells showed less spatial summation than those recorded from the internal horizontal cells.

3. Bipolar cell responses consisted of sustained potentials associated with an antagonistic centre-surround type receptive field (on-centre, off-surround or vice versa). Spike activity was not observed in bipolar cells.

4. Amacrine cells responded with transient depolarization both at the beginning and at the end of illumination. They sometimes showed spike activity. The amplitude of on- and off-depolarization showed slight dependence on stimulus geometry but a distinct centre-surround organization was not observed.

INTRODUCTION

In the vertebrate retina the detailed information received in the fine receptor mosaic is extensively processed in the subsequent neural network, and the ganglion cells consequently show a highly specialized behaviour. One direct way of analysing the function of the retinal network is to record

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from each element, starting from receptors, and comparing one class with the next.

In 1965, Tomita developed a micro-electrode technique for penetrating small retinal cells and succeeded in making intracellular recordings from single carp cones. We applied his technique to the cells located in the inner nuclear layer of the carp retina (Kaneko & Hashimoto, 1969). These were distinctly different from S-potentials and we suggested that the cells were either bipolar cells or amacrine cells as judged by their depth, localization of perikarya stained with intracellular dye injection, and their failure to respond to antidromic stimulation of the optic nerve. Dye injection is the most reliable present method of demonstrating histologically a cell penetrated with an electrode, but Niagara Sky Blue, which we used in the above work, failed to diffuse into the dendritic and axonic trees. Cells marked in this way can be accurately localized, but positive identification is not possible if cell bodies of more than one type are present in a given region such as the inner nuclear layer.

In 1968, Stretton & Kravitz developed a dye injection method capable of revealing the geometry of a cell down to very fine processes. The present paper describes an application of their method to the goldfish retina, with special reference to cells in the inner nuclear layer. Several types of cells were identified and correlations made between morphology and physiological responses.

#### METHODS

Goldfish were used instead of carp, since they were more readily available. Carp and goldfish belong to the same group, *Cyprinidae*, and no significant differences are expected in their retinal physiology. A fish was dark-adapted before the eye was removed and the retina isolated from the pigment epithelium. The whole retina was cut into four pieces of about equal size. Dye was injected into not more than one unit in each retinal quadrant to make it easier to find the cells histologically. The retina was mounted in a cooled chamber where moist oxygen gas mixed with 5% carbon dioxide was continuously supplied.

A two-channel photostimulator was made from a dissection microscope, using its two independent optical systems to provide the stimuli. Ophthalmoscope lamps, powered with a regulated direct current, were mounted against the ocular lenses so that the light travelled in a direction opposite to that of the light in a normally operated microscope. The light beams were made to converge on the retina. Stops in each light path gave a spot (0.2–4 mm diameter) or an annulus (1 mm i.d., 2 mm o.d.). Since the lamps were turned on or off electrically with slow rise and fall times, a precise description of the time course and latency of retinal responses could not be made. Usually white light was used; when red light was necessary, a Kodak Wratten filter No. 25 (orange red) or No. 89A (deep red) was interposed in the light path. Light intensity was controlled with neutral density filters and was usually about 2 log units above threshold. Experiments were done in subdued room light.

In a single vertical penetration of isolated goldfish retina different types of responses were successively recorded by advancing the electrode from one layer to the next. Penetration of a cell was signalled by a sudden drop of d.c. potential of

40–50 mV. Since the response was rarely maintained longer than several minutes, response characteristics of the cell could not be examined extensively. Nevertheless, with limited observation of their responses to a spot and an annulus or to white light and red, cells could be categorized physiologically into several groups on the basis of previous knowledge.

The dye injection method was slightly modified from that reported by Stretton & Kravitz (1968) so that it was applicable to smaller cells. Glass micropipettes, prepared by the fibreglass method developed by Tasaki, Tsukahara, Ito, Wayner & Yu (1968), were filled with 6% aqueous solution of Procion Yellow M4RS. The negatively charged dye was electrophoretically injected by passing a current of 5–10 nA for 1–2 min. When less current was used the dye did not come out of the pipette; if the current was too intense the cell was damaged and its response quickly lost. In about half of the successful injections the response was maintained after the injection.

After the injection the retina was kept in a moist chamber for 15–30 min to give the dye time to diffuse. Then the retina was fixed with a mixture of formaldehyde and glutaraldehyde at pH 4, embedded in Epon or Maraglas (Erlandson, 1964) and cut into serial sections of 10  $\mu$  thickness. Preparations were examined under the fluorescence microscope without counter-staining. The orange-yellow fluorescence of the dye was readily distinguished from the yellow-green background autofluorescence. As an injected cell usually appeared in several sections, it was reconstructed either in a montage photograph or by tracing with a camera lucida.

## RESULTS

Dye was injected into approximately 250 cells after their electrical responses were recorded. Thirty-eight of these cells were located in subsequent histological examination.

### *Horizontal cells*

Cajal (1955) described three types of horizontal cells in the teleost fish retina: external, intermediate and internal (cf. Text-fig. 4). They are located in separate and successive sublayers in the inner nuclear layer, the external horizontal cell in the most sclerad part, the intermediate in the middle, and the internal in the most vitread.

It was usual to record successively three to five S-potentials in any single penetration in any part of the retina. In the present study, however, only two types of horizontal cells were histologically identified, the external and the internal horizontal cell; the intermediate horizontal cell was not identified. By penetrating the retina from the receptor side, the S-potentials recorded most sclerad were localized to external horizontal cells. Plate 1 shows a montage of one of the fourteen successfully injected external horizontal cells. In this example, as in others, the polygonal perikaryon measures about 20  $\mu$  horizontally and lies in the most sclerad part of the inner nuclear layer. Several short dendritic processes extend from the sclerad surface of the cell body and can be traced up to the receptor terminal region. An axon comes out of the cell body at the corner between

the side wall and the vitread surface. It is single and almost uniform in diameter, and runs horizontally in about the same plane as the cell body for about  $250\ \mu$ ; no special terminal structure is observed. The same type of cell examined in horizontal sections appeared star-shaped, with short processes extending from the corners. By penetrating the retina from the vitreous side, the S-potentials recorded most vitread were localized to structures that appeared identical to Cajal's internal horizontal cell. Plate 2 shows one of ten internal horizontal cells successfully demonstrated. They are single more or less cylindrical structures about  $5\text{--}10\ \mu$  in diameter running horizontally in the middle part of the inner nuclear layer. In Plate 2 a thin extreme portion extends into a more sclerad part of the same layer, but does not appear to leave the inner nuclear layer. These cells appeared similar in both horizontal and vertical sections. In four of the ten stainings of these internal horizontal cells a Müller cell or part of one appeared also to have taken up stain. The dye in the Müller cell was considered to be an artifact due to leakage through the injection site, since a Müller cell was also found stained in a control experiment in which a large amount of dye was injected under circumstances in which neither resting potential nor response to light was recorded. Whenever the dye was injected in moderate amounts into an S-potential unit recorded vitread, it was limited to an internal horizontal cell. During several penetrations from the receptor side, injections were made of the second or third S-potential cells encountered. In some of those the external horizontal cell was stained, in others the internal horizontal cell.

In respect to spectral responses, two types of S-potentials are known to exist: the luminosity (L) type, responding with hyperpolarization to all wave-lengths of visible light, and the chromaticity (C) type, changing its response polarity according to wave-length (Wagner, MacNichol & Wolbarsht, 1960). In the present study the type of S-potential was identified by illuminating the retina with white and red light alternately. An attempt was made to correlate the type of S-potential with a particular type of horizontal cell, but no consistent correlation was found; both L- and C-type S-potentials were correlated with external horizontal cells and also with internal horizontal cells.

One physiological difference between the two histological types of horizontal cell was in the influence of spot size on response amplitude: the response from external horizontal cells showed less spatial summation than that from the internal horizontal cell. As an index of the amount of spatial summation the ratio of response amplitude to large ( $2.8\text{ mm}$  diameter) and small ( $1\text{ mm}$  diameter) light spots was calculated for each cell. The ratios were then determined for successively recorded horizontal cells in a number of different penetrations. The comparison of amplitude

ratios was always made on pairs of cells recorded in a single penetration, since the amount of spatial summation could vary in the same type of cells located in different parts of the retina. For similar reasons an L-type unit was compared with another L-type unit obtained in the same penetration, and a C-type unit with another C-type unit. In Text-fig. 1 open

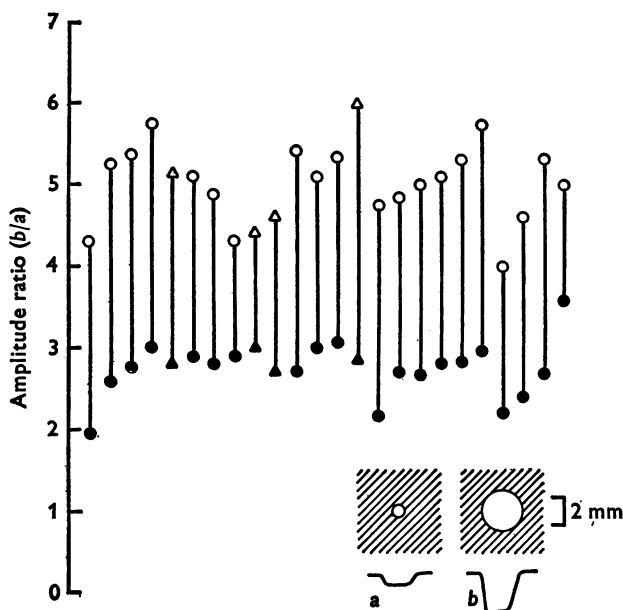


Fig. 1. Comparison of the external and the internal horizontal cells in their amount of spatial summation. A symbol gives the ratio of response amplitude to a large spot (2.8 mm diameter) to that to a small spot (1 mm diameter) of almost equal intensity. Symbols represent the following responses: ○ L-type from an internal horizontal cell, ● L-type from an external horizontal cell, △ C-type from an internal horizontal cell, ▲ C-type from an external horizontal cell. A pair of symbols connected with a vertical line are the units obtained in a single vertical penetration of the retina.

symbols representing the internal horizontal cells always show larger values than filled ones representing the external horizontal cells. The vertical connecting lines indicate that pairs of points were obtained in single vertical penetrations. The above relation was consistent for both L- and C-type units.

### *Bipolar cells*

In the carp, units were seen which responded with sustained potentials and had antagonistic centre-surround type receptive fields (Kaneko & Hashimoto, 1969). Similar cells were found in the goldfish and were identified as bipolar cells. Sixteen off-centre cells and three on-centre cells were injected.

Plate 3 is a photomicrograph of an off-centre cell and Plate 4 is a montage photograph of an on-centre cell. It is interesting that there is no morphological difference between on-centre and off-centre cells. The perikaryon lies in the middle part of the inner nuclear layer; the dendritic stem runs sclerad, branching in the external plexiform layer, and the dendritic twigs end at the level of the receptor terminals. The dendritic field measures about  $40\ \mu$  in diameter. The axon runs down to the inner

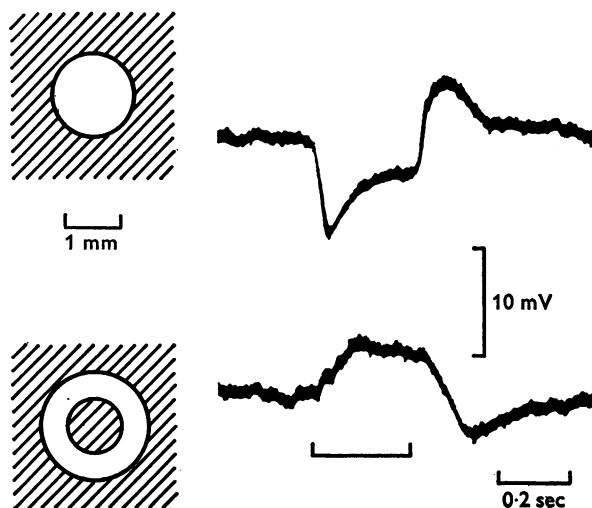


Fig. 2. Intracellular recordings from a bipolar cell which is hyperpolarized by a spot (upper tracing) and depolarized by an annulus (lower tracing). Horizontal line below the response traces indicates roughly the period of illumination. Both types of illumination are white light of about equal intensity. D.c. recording, positivity upwards.

plexiform layer where it ends as a conical or bulbous terminal knob which is almost as large as the perikaryon. Small numbers of fine short processes originate from the terminal knob and run horizontally about  $5\text{--}10\ \mu$ . Cajal (1955) described two kinds of morphologically different bipolar cells, a rod bipolar cell with large perikaryon, enormous dendritic branches and a terminal knob, and a cone bipolar cell with a smaller perikaryon, fewer dendrites and no terminal knob (Text-fig. 4, rb and cb). Cajal's interpretation of fish bipolar cells was recently modified by Stell (1967), who showed that the 'rod bipolar cell' has synaptic contacts with both rods and cones while the 'cone bipolar cell' has contacts exclusively with cones. The bipolar cells revealed by dye injection in this study correspond to Cajal's 'rod bipolar cells'.

Text-figure 2 shows the response of a bipolar cell that was hyperpolarized by a spot and depolarized by an annulus. Bipolar cells behaving in the

reverse manner (on-centre, off-surround) were also observed. Most bipolar cells had centre-surround type receptive fields, but three identified by dye injection responded only with depolarization either to a spot or to an annulus. The possibility remains, however, that the stimulus size was not adequate to reveal the centre-surround arrangement. Records like those of Text-fig. 2 were similar to intracellular recordings from ganglion cells, except that all-or-none spikes were not observed. Injury discharges, which are expected in cells that generate spikes, were not recorded in these units.

An oscillatory potential similar to that described by Kaneko & Hashimoto (1969) was observed in some of the above bipolar cells. As the oscillatory potential was more frequently observed when the preparation was aged, it may be an abnormal phenomenon. These oscillations, nevertheless, seem to be peculiar to bipolar cells and merit further study.

### *Amacrine cells*

We have previously described units that were transiently depolarized both at the beginning and at the end of illumination (Kaneko & Hashimoto, 1969). Similar types of cells were found in the goldfish and identified as amacrine cells.

Six amacrine cells were successfully injected. Plate 5*a* is a montage photograph of one of these made from four serial sections, and Plate 5*b* is a more complete reconstruction of the same cell traced with a camera lucida. The perikaryon measures about  $20\ \mu$  in diameter and lies in the vitread part of the inner nuclear layer. A short stout dendritic stem comes out from the vitread side of the perikaryon and branches several times into smaller processes. The whole dendritic field measures about  $350\ \mu$ . No axon-like process was found in amacrine cells.

Text-fig. 3 shows an example of the amacrine cell response. The amplitude of on-depolarization and that of off-depolarization showed slight dependence on stimulus geometry but there was no distinct centre-surround organization, such as that found in a bipolar cell. Nine out of twenty-five amacrine cells showed spike activity superimposed on slow potentials (upper tracing, Text-fig. 3).

### DISCUSSION

Dye injection from the recording pipette is the most reliable present method for identifying a cell recorded intracellularly. The method is especially useful in a tissue such as the retina in which one must penetrate cells without visual control. Various kinds of dyes have been used for this purpose, but Procion Yellow is superior to others in many respects. It

fulfils most of the requirements for correct localization of recording site, for example, the response was maintained after injection of the dye in about half of the examples of these experiments, indicating that the electrode stayed in the same cell during dye injection. The greatest advantage of this dye is that it can reveal a cell in its entirety, whereas other dyes remain confined to the site of electrode penetration.

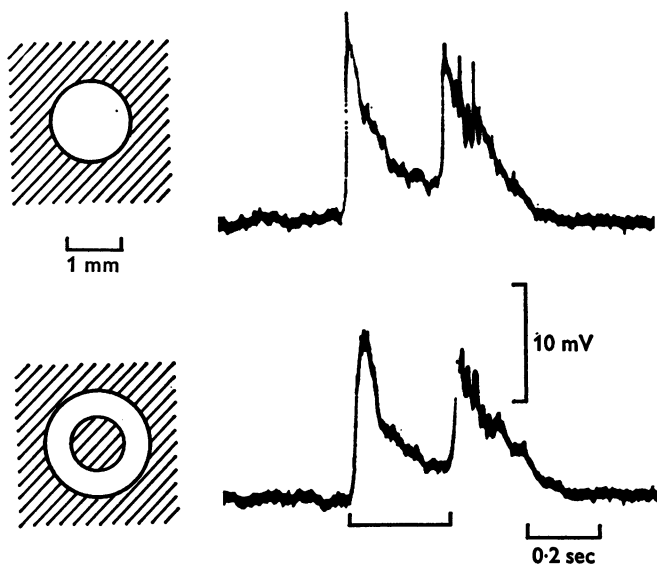


Fig. 3. Intracellular recordings from an amacrine cell which is transiently depolarized both at the beginning and at the end of illumination. Horizontal line below the response traces indicates roughly the period of illumination. The other experimental conditions are the same as in Fig. 2.

Horizontal cells have previously been implicated as the origin of S-potentials (MacNichol & Svaetichin, 1958; Mitarai, 1958; Oikawa, Ogawa & Motokawa, 1959). In the present study S-potentials were localized in the external and internal horizontal cells. In a single penetration of the retina several S-potential units were recorded one after another. The number of successive units was usually two or three, occasionally up to five. It seems clear on histological grounds that each of the two cell types occupies more or less separate sublayers. However, an external horizontal cell is often dislocated vitread, and several internal horizontal cells are usually found stacked above one another, so that more than one of each type can at times be recorded in a single vertical penetration. Consistent with this interpretation, all of the identified horizontal cells were either external or internal in type even though injections were made irrespective of vertical location.

In the goldfish, Stell (1967) has demonstrated histologically that the external horizontal cell receives synaptic input from cones and the intermediate horizontal cell from rods. His finding seems to provide a reasonable interpretation for the fact that intermediate horizontal cells were not demonstrated in this study. Presumably the retina was maintained in the photopic state, so that the rods were saturated and no rod influence was forwarded to the intermediate horizontal cell upon illumination.

All of the bipolar cells revealed by dye injection were rod bipolar cells, so designated by Cajal. This may have resulted from a biased selection at penetration, since a rod bipolar cell has a larger cell body than a cone

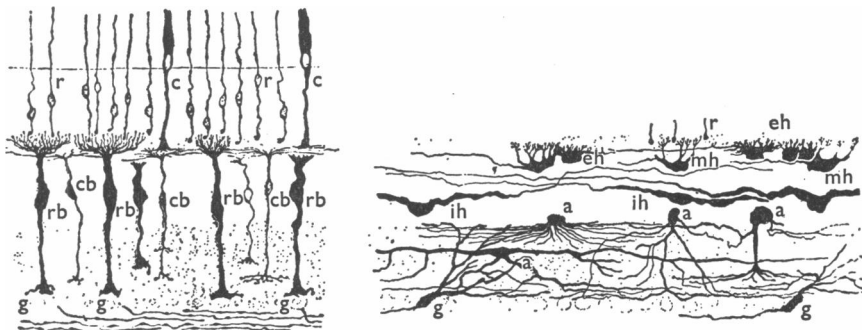


Fig. 4. Vertical section of the teleost retina stained by the Golgi method. Taken from Cajal (1955). The left section shows the vertical elements, the most direct pathway from receptors to ganglion cells. The right section shows the horizontal elements, the route of lateral connexions within the retina. c, cones; r, rods; cb, cone bipolar cells; rb, rod bipolar cells; eh, external horizontal cells; mh, intermediate horizontal cells; ih, internal horizontal cells; a, amacrine cells; g, ganglion cells.

bipolar cell. As rod bipolar cells receive both rod and cone inputs according to histological observation by Stell (1967), it seems reasonable to assume that a rod bipolar cell remains functioning both in the scotopic and in the photopic states.

Cajal (1955) described, in the primate retina, that the horizontal cell axon ends on a remote receptor terminal and suggested that this might also be true of fish retina. In fish retina, however, horizontal cell axons have so far not been found by electron microscopy. Axons revealed by dye injection in some external horizontal cells were not traced up to the receptor terminal so that the structures with which these axons synapse still remain uncertain.

Recently Remler, Selverston & Kennedy (1968) and Payton, Bennett & Pappas (1969) observed in crayfish axons that a small amount of Procion Yellow diffused from an injected cell to an adjacent one across tight junctions where electrical transmission is known to exist. In the

present study the dye injected into an external or internal horizontal cell was limited in a single cell, even though tight junctions are found in large numbers between these cells (Yamada & Ishikawa, 1965; Stell, 1967). This finding may not necessarily conflict with the hypothesis that the lateral connexions of horizontal cells are electrotonic (Naka & Rushton, 1967), since the possibility remains that the amount of injected dye was too small to give any detectable diffusion or that tight junctions of horizontal cells are less permeable to large molecules.

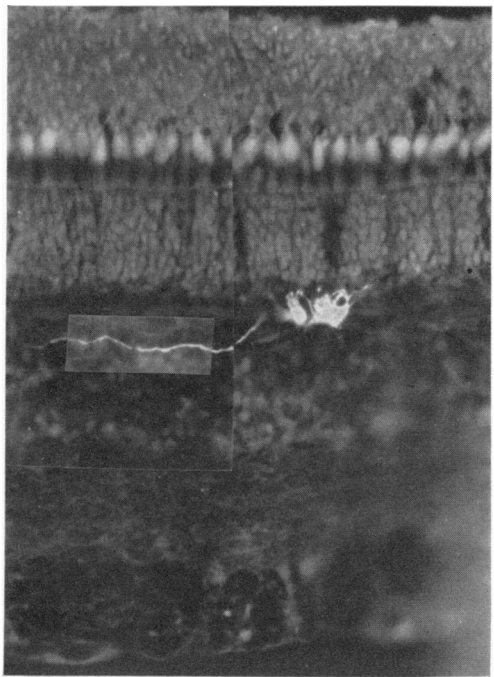
Bipolar cells were shown to respond with sustained potentials and to have antagonistic centre-surround type receptive fields. Amacrine cells were identified as the origin of transiently depolarizing potentials at light 'on' and 'off'. These findings on bipolar and amacrine cells are in good accord with results in the mudpuppy (Werblin & Dowling, 1969).

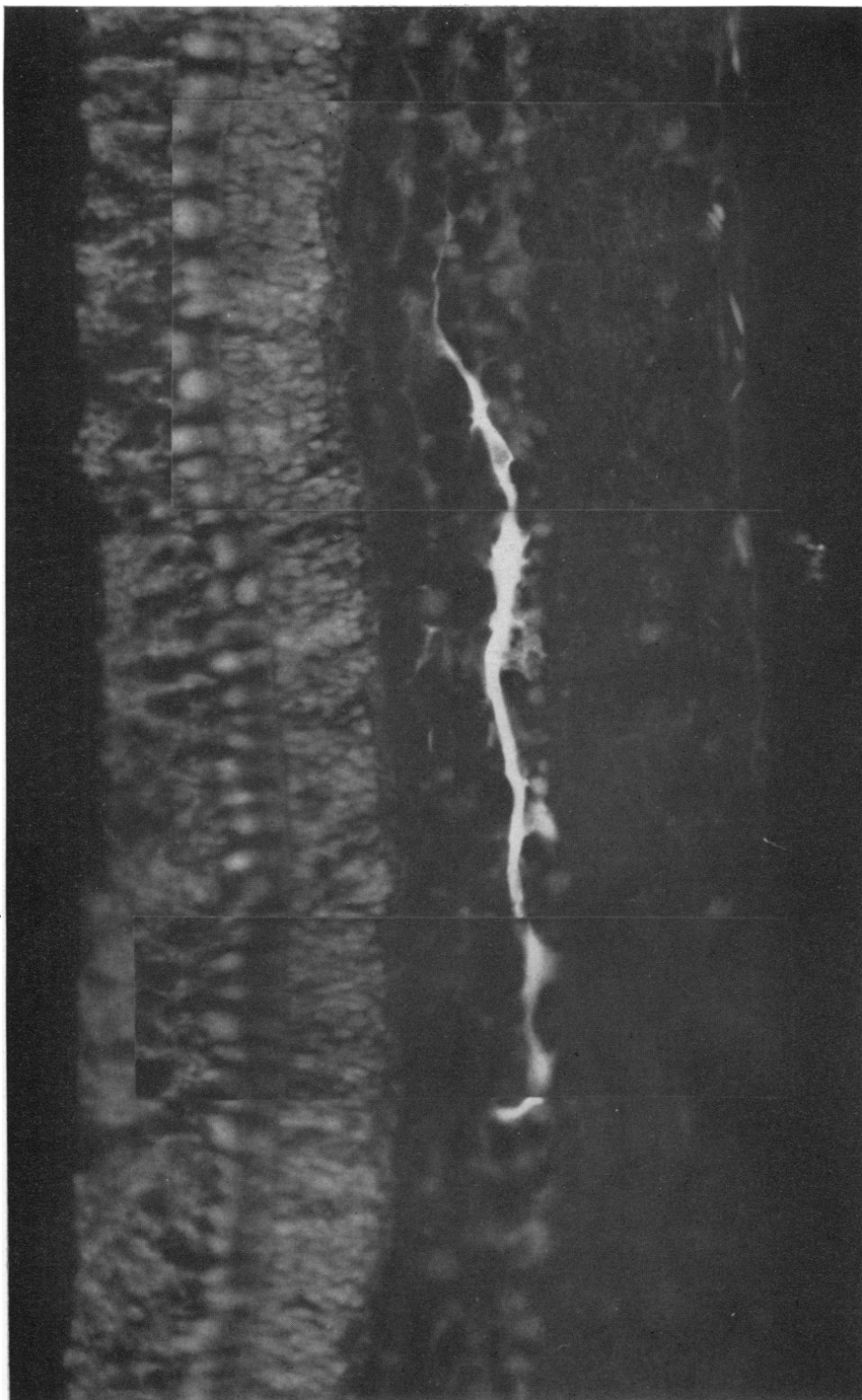
While correlations were established between morphology and response type for many retinal cells, a more detailed analysis will be necessary for a full interpretation of the functional wiring diagram of retinal cells.

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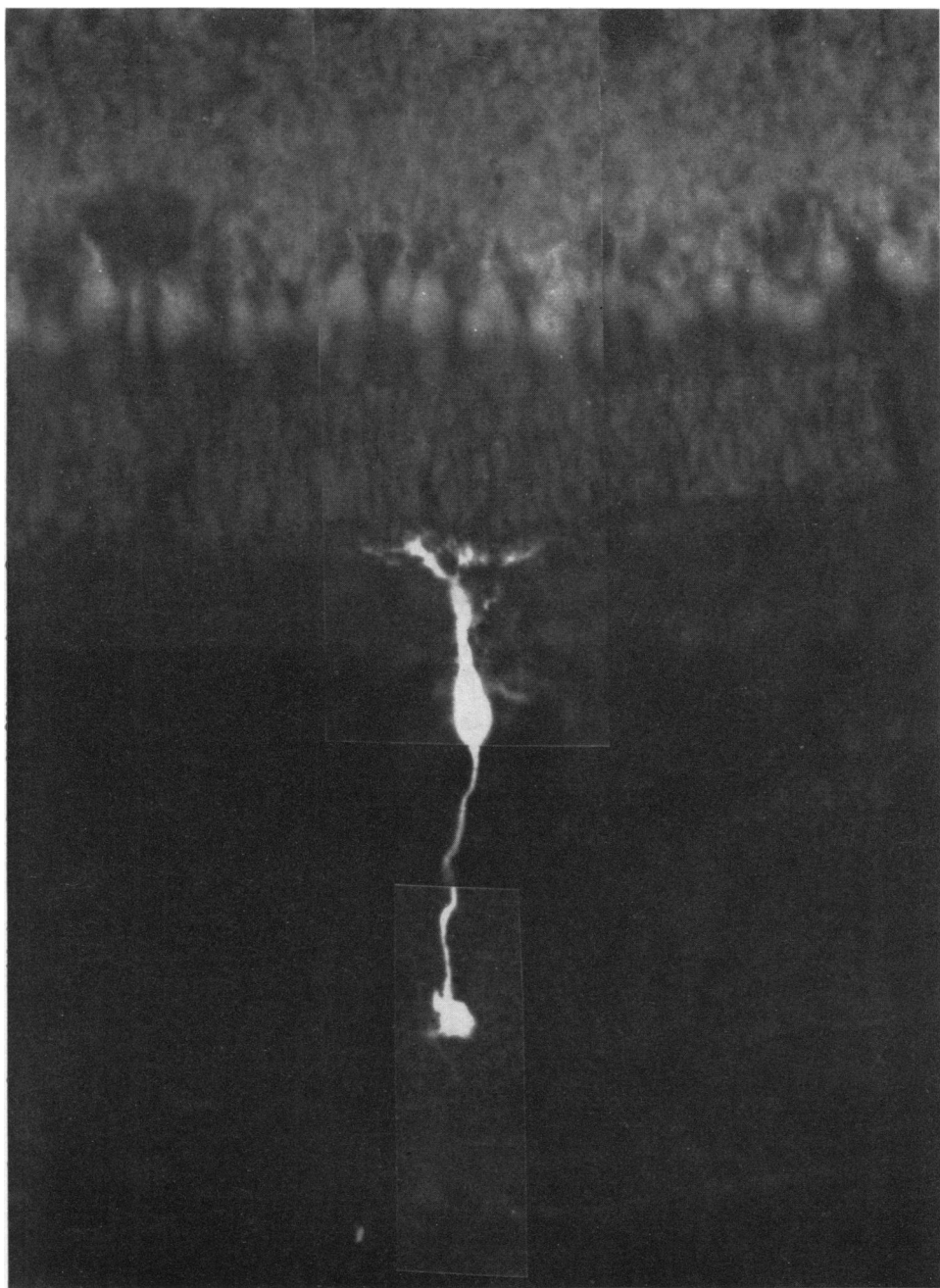
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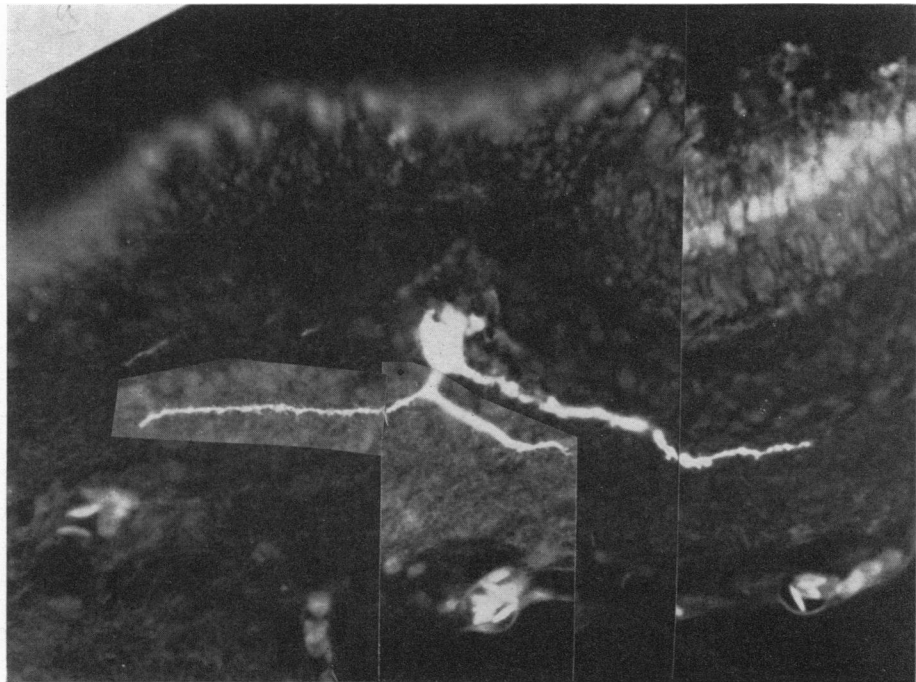


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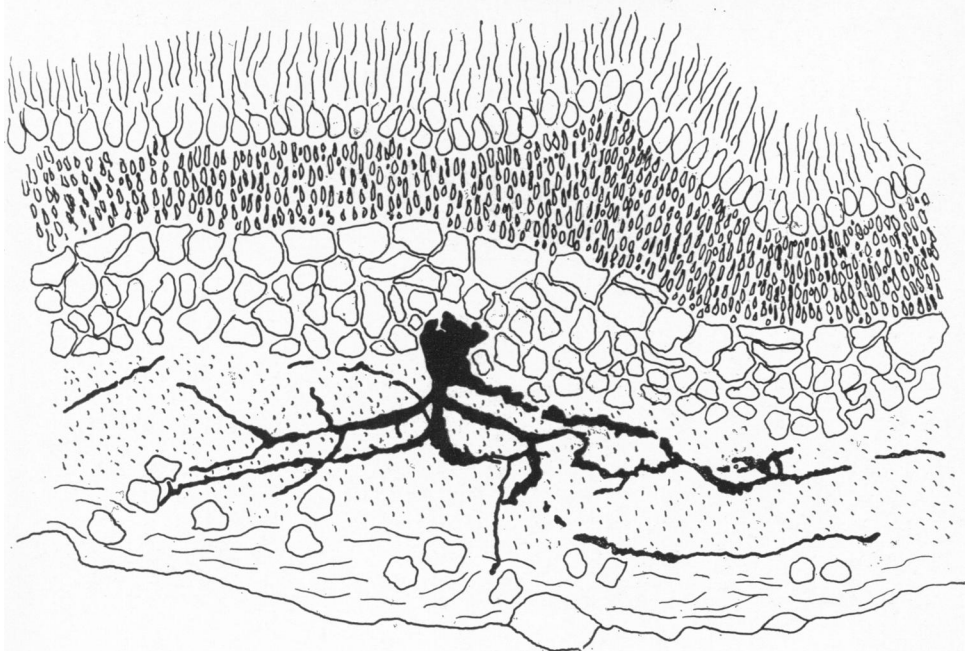




AKIMICHI KANEKO



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AKIMICHI KANEKO

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## EXPLANATION OF PLATES

## PLATE 1

A montage photomicrograph of an external horizontal cell in which an S-potential, located sclerad, was recorded.

## PLATE 2

A montage photograph of an internal horizontal cell in which an S-potential, located vitread, was recorded.

## PLATE 3

A successfully injected bipolar cell in which the same type of response as shown in Text-fig. 2 (off-centre, on-surround) was recorded.

## PLATE 4

A montage photomicrograph of another bipolar cell which has an on-centre, off-surround receptive field.

## PLATE 5

An injected amacrine cell from which a response similar to that shown in Text-fig. 3 was recorded.

*a*, a montage photomicrograph from four serial sections;

*b*, a complete reconstruction of the same cell traced with a camera lucida.